Short communication

The translation-enhancing region of the Semliki Forest virus subgenome is only functional in the virus-infected cell

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We have recently shown that the Semliki Forest virus (SFV) subgenome contains a translation enhancer region in the coding part of its RNA. The enhancer increases the translation of the viral structural genes approximately ten-fold and thereby plays a key role in virus assembly. In this study we conclusively show that this translation enhancer represents an adaptation to the

The alphavirus Semliki Forest virus (SFV) is a positive-stranded RNA virus which replicates in the cytoplasm of its host cells (Strauss & Strauss, 1986). In a newly infected cell, the 5' two-thirds of the viral genome is directly translated into four non-structural proteins. These will replicate the genome via an intermediate of negative polarity and also transcribe the 5' one-third of the intermediate into a subgenomic RNA species, which is used for translation of the viral structural proteins. Most cells have developed systems to detect viral infection and to minimize its spread. One cellular strategy is to inactivate the translation machinery and thereby obstruct the synthesis also of viral proteins. Alphaviruses, which trigger this kind of defence, have adapted to the restricted environment and manage to translate their subgenomic mRNAs into proteins in a strikingly efficient manner. The overall result is a selective shut down of host- but not of virus-specific protein synthesis, which is effective from approximately 4 h after infection (Schneider & Shenk, 1987).

The exact molecular basis of decreased synthesis of host-specific molecules is still unclear, as is the basis of continued virus-specific translation (reviewed by Strauss & Strauss, 1994). However, these appear to stem from the combined effect of several different events. Upon infection the intracellular concentration of potassium ions is decreased and the concentration of sodium ions is increased. This results in a preferential translation of viral mRNAs (Carrasco, 1977; Garry, 1994; Garry et al., 1979). Alphavirus-infected cells also display decreased levels of functional eIF-4B giving poor translation of most cellular mRNAs. In contrast, SFV subgenomes are readily translated when only limited amounts of eIF-4B are present (Berben-Bloemenheuvel et al., 1992; Kozak, 1994). The remarkably efficient translation of the SFV subgenome is explained both by the structure of its 5'-untranslated region (5'-UTR; see Kozak, 1994 and Berben-Bloemenheuvel et al., 1992) and by the existence of a translation enhancer region in the beginning of the coding part of the molecule. The subgenomic RNA molecule is capped, has a 51 base 5'-UTR followed by one open reading frame (ORF) encoding the viral structural proteins in the order C–p62–E1 (Garoff et al., 1982). The enhancer is within the first 102 bases of the coding part of the C gene and will increase the translational efficiency of any gene, provided that it is inserted downstream of and in the same ORF as the enhancer segment (Sjöberg et al., 1994). Frolov & Schlesinger (1994) have shown that a similar enhancer segment is present also in Sindbis virus, and that it is functional only in a virus-infected cell. In this work we confirm the latter result and more stringently show that the translational enhancer of the gene encoding the C protein (the C gene) represents an adaptation to the environment in the virus-infected cell.

To measure whether the translational enhancer is functional in uninfected cells we constructed a series of plasmids in which recombinant SFV subgenomes were placed directly under the control of the SP6 promoter. The recombinant subgenomes that are transcribed from these plasmids contain the complete 5'-UTR and either 0, 57 or 144 5' bases from the C gene, followed by the β-galactosidase gene lacZ (SFV3nr-lacZ, SFVα2nr-lacZ and SFVb7nr-lacZ, respectively) or the complete C gene followed by lacZ (SFVCnr-lacZ). Transfections with these recombinant subgenomes will give rise to β-galactosidase and three fusion proteins (α2-β-galactosidase, b7-β-galactosidase and C-β-galactosidase, respectively).
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Fig. 1. Schematic drawings of the in vitro transcribed RNA molecules used in this study. The first RNA molecule (SFV) shows the wild-type SFV subgenome. The RNA molecules 2–5 encompass the complete non-structural region of SFV, the subgenomic promoter, the complete 5'-UTR of the subgenome and 0, 57, 144 or 801 5' bases from the C protein gene followed by the β-galactosidase gene lacZ. The 801 base insert corresponds to the complete C protein gene. The RNA molecules 6–9 encompass the complete 5'-UTR of the subgenome followed by 0, 57, 144 or 801 5' bases from the C protein gene and lacZ. All RNA molecules carry a 5' m7G(5')ppp(5')G group (CAP) and a poly(A) tail (Am). The 'nr' in the name of the molecules 6–9 refers to the fact that they are not able to replicate.

respectively). Fig. 1 summarizes all RNA molecules used in this study.

Our approach to measure the translational efficiency of recombinant SFV subgenomes was to (i) transfect BHK-21 cells with in vitro synthesized mRNA by electroporation (Liljestrom & Garoff, 1991; Suomalainen & Garoff, 1994), (ii) plate the cells and metabolically label all newly synthesized proteins with [35S]methionine, (iii) lyse the cells with detergent and (iv) extract the proteins of interest with antibodies and analyse the immune complexes by SDS-PAGE (Laemmli, 1970). Preliminary experiments where transfected cells were stained for β-galactosidase in situ (Sanes et al., 1986), showed that the intracellular level of β-galactosidase was maximal at 4 h post-transfection. We therefore chose 3 h post-transfection as a suitable time to measure translational efficiencies. The fraction of cells transfected (the transfection efficiency) was measured by β-galactosidase staining in situ (Sanes et al., 1986) and found to be higher than 90% in all cases. To obtain correct quantifications it was necessary to establish conditions for complete solubilization of the fusion proteins. To this end aliquots of BHK-21 cells were transfected with replication-competent recombinant SFV genomes encoding β-galactosidase, a2-β-galactosidase, b7-β-galactosidase or C-β-galactosidase (SFV3-lacZ, SFVa2-lacZ, SFVb7-lacZ and SFVC-lacZ, respectively) (Sjöberg, 1994). At 30 hours post-transfection parallel samples of each transfection were lysed in NP40- or SDS-containing lysis buffer (Sjöberg et al., 1994) and analysed by SDS–PAGE followed by Coomassie blue staining. This showed that only C-β-galactosidase required SDS in order to become completely solubilized (data not shown). We applied the SDS lysis method to experiments involving the C-β-galactosidase fusion protein and NP40 lysis to all other experiments. Another key step in our quantifications was immunoprecipitation. Quantitative extraction of all forms of β-galactosidase requires that the epitopes used by the antibodies are accessible on all fusion proteins. We compared a set of different antibodies for their ability to precipitate β-galactosidase and the fusion proteins; (i) a monoclonal mouse anti-β-galactosidase antibody (Boehringer-Mannheim catalogue number 1083 104) and (ii) a polyclonal rabbit anti-SFV C protein antibody. The antibodies were raised against SDS-denatured SFV C protein; (H. Garoff, unpublished results) together with the monoclonal used in (i) and (iii) a polyclonal mouse
Fig. 2. Translation of recombinant SFV subgenomes in BHK-21 cells (lanes 1–5) and in rabbit reticulocyte lysates (lanes 6–8). Aliquots of BHK-21 cells were transfected by electroporation (Suomalainen & Garoff, 1994) of in vitro transcribed (Liljeström et al., 1991) SFV3nr-lacZ or SFVb7nr-lacZ RNA (lanes 1–2) or with SFV3nr-lacZ and SFVb7nr-lacZ (lane 3) or with SFV3-lacZ or SFVb7-lacZ (lane 4 and 5, respectively) RNA. The volumes of transcription mixture used were 40 µl (lanes 1 and 2), 40 µl + 40 µl (lane 3) or 20 µl (lanes 4 and 5). The RNA concentration in the transcription mixtures were equal, as judged by ethidium bromide staining in agarose gels. The translation efficiency was checked by β-galactosidase staining in situ (Sanes et al., 1986) at 4 h (SFV3nr-lacZ or SFVb7nr-lacZ) or 20 h (SFV3-lacZ or SFVb7-lacZ) post-transfection and was found to be > 90 %. At 2.5 h (lanes 1–3) or 7.5 h (lanes 4 and 5) post-transfection the cells were starved of methionine for 30 min, pulse-labelled with [35S]methionine (3.7 MBq/dish) for 15 min, chased in an excess of unlabelled methionine for 15 min and then solubilized in NP40-lysis buffer supplemented with 0.2 mM-PMSF, 0.1 mM-N-ethylmaleimide and 1 µg/ml pepstatin A, as described (Sjöberg et al., 1994). For the in vitro translation experiment SFV3nr-lacZ (lane 6), SFVb7nr-lacZ (lane 7) or a 1:1 mixture of the two RNAs (lane 8) were translated in the presence of [35S]methionine (1 mM) in rabbit reticulocyte lysate. The translation was carried out in a volume of 7.5 µl made up by 0.75 µl transcription mixture (Liljeström et al., 1991), 1.0 µl of a solution containing 1 mM-MgCl₂ and 70 mM-KCl pH 6.5, 3.0 µl rabbit reticulocyte lysate and 2.75 µl translation cocktail containing 1.1 Mβ₂M, 0.27 mM-potassium acetate, 0.11 mM-spermidine-HCl. 0.14 mM each of 19 amino acids (no methionine), 2.7 mM-ATP, 0.54 mM-GTP and 27 mM-creatine phosphate and continued for 30 min at 30 °C. Cell lysates were either subjected to immunoprecipitation (Wahlberg et al., 1989) with a polyclonal mouse anti-β-galactosidase antibody (Sigma G-4644; lanes 1–5) and the immune complexes then analysed by SDS-PAGE or separated directly by SDS-PAGE (lanes 4–8) in 7 % acrylamide gels. The equivalent of 2.4 x 10⁴ cells/well (lanes 1–3) or 1.2 x 10⁴ cells/well (lanes 4 and 5) or 0.3 µl translation mixture (lanes 6–8) were applied. Shown are autoradiograms of the dried gels after 7 days (lanes 1–3) or 1.5 h (lanes 4–8) of exposure.

anti-β-galactosidase antibody (Sigma catalogue number G-4644). All three antibody sets proved to be equally effective (data not shown). Furthermore, it was necessary to establish that the epitopes used were stable also after lysis in SDS. We therefore lysed parallel samples of cotransfected cells (SFV3nr-lacZ + SFVb7nr-lacZ) with NP40 and SDS, respectively, and compared the ability of the polyclonal anti-β-galactosidase antibodies to immunoprecipitate β-galactosidase fusion proteins from the two samples. The antibodies were found to recognize proteins solubilized with either detergent equally well (data not shown). Taken together these control experiments showed that it is possible to accurately measure the amount of β-galactosidase and β-galactosidase fusion proteins in BHK-21 cells transfected with recombinant SFV subgenomes. We then compared the production of β-galactosidase-containing proteins in cells transfected with SFV3nr-lacZ or SFVb7nr-lacZ. Fig. 2 (lanes 1 and 2) shows that equal amounts were produced in both cases. The corresponding results are shown in Table 1. To ascertain that the two mRNAs would encounter the same environment, cells were co-transfected with a 1:1 mixture of SFVb7nr-lacZ and SFV3nr-lacZ RNA. As the transfection efficiency in all one-RNA transfections was higher than 90 %, at least 81 % of the cells should contain both RNA types in a two-RNA co-electroporation. The production of β-galactosidase and b7-β-galactosidase was indistinguishable also under these more stringent testing conditions (lane 3 and Table 1). The effect of the translation enhancer has been shown before (Sjöberg et al., 1994) and is demonstrated in Fig. 2 (lanes 4 and 5) where the pattern of protein synthesis in cells that have been transfected with recombinant SFV genomes (SFV3-lacZ and SFVb7-lacZ, respectively) is shown as a control. In these genomes the viral structural proteins (C–p62–E1) have been replaced by the β-galactosidase gene lacZ, (SFV3-lacZ, lane 4) or by the 144 base segment containing the translation enhancer, followed by lacZ (SFVb7-lacZ, lane 5). The increase in b7-β-galactosidase synthesis induced by the translation enhancer (compare lanes 4 and 5) is approximately eight-fold (Sjöberg et al., 1994).

To rule out the possibility that differences in mRNA stability could influence the level of protein production, cells were transfected with pSFV3nr-lacZ or pSFVb7nr-lacZ subgenomes, pulse-labelled at 2, 3, 4, 5 and 6 h post-transfection and the amount of β-galactosidase and b7-β-galactosidase was quantified as described in the legend of Table 1. Even though the amount of protein synthesized decreased with time, their relative levels remained constant (data not shown). We also repeated the experiments with a different pair of recombinant subgenomes; SFVa2nr-lacZ and SFVCnr-lacZ. The former subgenome carries 57 5' bases from the C gene and will consequently promote low level a2-β-galactosidase production in SFV-infected cells. The SFVCnr-lacZ carries a complete C gene upstream of lacZ and consequently promotes high level C-β-galactosidase production in SFV-infected cells. However, in the non-replicating versions all recombinant subgenomes tested were found to promote comparable levels of protein production (summarized in Table 1) both in single RNA and in mixed RNA transfections.

As a complement to the metabolic labellings, enzymatic β-galactosidase activity (Silhavy et al., 1984) in
Table 1. Protein production from recombinant SFV subgenomes

Recombinant proteins were produced in BHK-21 cells transfected with recombinant SFV subgenomes (set up A and B) and in rabbit reticulocyte lysate (set up C) and separated by SDS-PAGE (see the legend of Fig. 2 for details). The amount of radiolabel in each band was quantified by the Fujix Bio-Image analyser system Bas 2000 (Fuji Photo Film). The data presented were corrected for differences in methionine content and normalized to the level of b7-fl-gal (set up A and C) or C-fl-gal (set up B) production in a single mRNA transfection (or translation) as indicated by an asterisk (*). The labelled cells were solubilized in NP40 lysis buffer (A) or SDS-lysis buffer (B) prior to electrophoresis.

<table>
<thead>
<tr>
<th>Set up</th>
<th>mRNA</th>
<th>Protein</th>
<th>Relative protein production</th>
<th>Mean</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>A</td>
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<td>12</td>
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<tr>
<td></td>
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<td>b7-β-gal</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>3nr-lacZ + b7nr-lacZ</td>
<td>β-gal</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b7-β-gal</td>
<td>6.6</td>
<td>6.6</td>
</tr>
<tr>
<td>B</td>
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<td>a2-β-gal</td>
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<tr>
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<tr>
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<td>3nr-lacZ + Cnr-lacZ</td>
<td>β-gal</td>
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<td></td>
<td>C-β-gal</td>
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<td></td>
<td>b7-β-gal</td>
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<td>5.1</td>
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</table>

NP40 lysates of transfected cells was measured. The β-galactosidase activities were comparable in lysates from cells transfected with SFV3nr-lacZ or SFVb7nr-lacZ, both at 3 h post-transfection (0.55 and 0.58 arbitrary units, respectively) and at 5 h post-transfection (0.60 and 0.66 arbitrary units, respectively).

A different approach to compare translational efficiency that allows a precise control of the mRNA mixing conditions is translation in cell-free systems. Aliquots of SFV3nr-lacZ and SFVb7nr-lacZ RNA were translated in a rabbit reticulocyte lysate-based translation system (Suomalainen et al., 1990) in the presence of [35S]methionine for 30 min at 30 °C and analysed by SDS-PAGE and fluorography (Fig. 2). Both SFV3nr-lacZ (lane 6) and SFVb7nr-lacZ (lane 7) resulted in a single major translation product each (β-galactosidase and b7-β-galactosidase) produced in comparable amounts. When the two mRNAs were mixed in a 1:1 ratio and then translated (lane 8), their translation efficiencies were still equal to each other. The results of four independent experiments are summarized in Table 1. In control experiments samples of the translation mixture were withdrawn and analysed also at earlier time points (after 2, 4, 8, 15 and 20 min of translation). This showed that, after an initial lag period where no translation products could be detected, equal amounts of the two proteins were produced at each time point (not shown).

In a recent paper Frolov & Schlesinger (1994) have shown that Sindbis virus also possesses a translation enhancer sequence in the 5' end of its C gene and that the enhancer is only functional in the virus-infected cell. The Sindbis virus enhancer sequence is substantially longer than that of SFV. In order to be effective some 220 bases downstream of the initiation codon are necessary (as compared to 102 bases in the case of SFV). The effect of the translation enhancer in uninfected cells was measured in BHK-21 cells that had been transfected with a plasmid where a DNA copy of a recombinant Sindbis virus subgenome encoding the β-galactosidase gene lacZ, either with or without the translation enhancer, had been placed under the control of a cytomegalovirus promoter. At 40 h post-transfection, cells were pulse labelled with [35S]methionine, lysed with SDS and β-galactosidase production was measured by immunoprecipitation and SDS-PAGE. The amount of recombinant RNA in the cells was checked in parallel samples that were lysed in RNAzol B (a phenol-based RNA purification cocktail) and analysed by northern blots. In this system transcription takes place in the nucleus and the mRNA is then transported to the cytoplasm where translation occurs. The RNAzol B lysis method, however, does not discriminate between nuclear and cytoplasmic RNA. The measured RNA levels therefore represent the total amount of recombinant Sindbis virus subgenomes in the cell and not necessarily the fraction available for translation. In this work, recombinant SFV subgenomes were transfected directly into BHK-21 cells and their ability to direct protein synthesis was compared. A series of control experiments ensured that all forms of β-galactosidase produced were quantitatively solubilized and immunoprecipitated prior to quantification. Fur-
thermore, we have also used a cell-free translation system and showed that the first 144 bases of the gene encoding the C protein do not change the rate of mRNA translation. These experiments unequivocally show that the translation enhancer sequence is only functional in an SFV-infected cell. The mechanism of action of the translation enhancer is still unclear. It has been suggested that a stem-loop structure, that is predicted within the first 100 bases downstream of the initiation codon, is involved. The stem-loop is thought to stabilize the ribosome initiation complex and thereby give it a better chance to bind active initiation factors (Frolov & Schlesinger, 1994), but this remains to be shown.

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References


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